Engineering Tough Materials: Biomimetic Eggshell

Final Report, 29 August 2016

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Abstract

This final report summarizes the work completed on the two year pilot project, which concluded 31 July 2016. The first section summarizes the two previous reports to place the current work in context. Two new research results sections are included here. The first concerns the use of a synthetic peptide, mimicking natural eggshell protein believed to be influential in biomineralization, to examine its effect on $CaCO_3$ formation, including a polymer-induced liquid precursor (PILP) mineralization. The second examines the interesting role of the eggshell membrane in shell fracture, demonstrating that it is intimately mechanically connected to the shell long after shell formation and mineralization. The report concludes with a brief outlook, including next steps to pursue in the new cooperative research arrangement between ERDC and the University of Cambridge.

Summary of Previous Reports

Preliminary report of 30 January, 2015

The preliminary report had two parts. First, the literature on biomimetic calcite synthesis was reviewed, with an aim of establishing gold-standard techniques for making biomimetic eggshell in large quantities. The literature was found to be surprisingly uniform in that most controlled studies of calcite biomineralization have utilized a vapor diffusion technique, where calcium is in solution and carbon and oxygen atoms or ions are introduced as a gaseous phase. Although well controlled, this process is slow and produces only small quantities of material. As such, the evidence supports our continuing with solution-based calcite synthesis with a mind towards scale-up of material synthesis for eggshell-like material in large quantities. Second, the results of a series of preliminary experiments on this project were presented, comparing calcitic materials produced in our laboratory with natural eggshell. Spectroscopy was utilized to examine the mineralization of calcite and the presence of amorphous calcium carbonate in the calcite matrix. Thermal analysis was used to establish the presence of organic materials within calcium carbonate.

Intermediate report of 30 June, 2016

This intermediate report covered work done across several areas contributing to our understanding of eggshell structure-properties relationships with a view towards biomimicry. First the structure and mechanical properties of different types of eggshell were compared across species. Second the entrapment of organic molecules in inorganic calcite was explored. The kinetics of calcite growth in the presence and absence of organic molecules was considered using serial real-time Fourier transform infrared spectroscopy. Finally preliminary work towards an electrospun artificial eggshell membrane was reviewed.

Biomimetic Eggshell Mineralization with a Synthetic Peptide

Eggshell biomineralization is known to be regulated by organic molecules such as proteins. The role of these organic molecules, however, is yet to be deciphered. On the one hand, it is nearly impossible to produce bulk $CaCO_3$ structures without using organic additives. On the other hand, using organic molecules extracted from biominerals, such as eggshells, has been proved to be inefficient towards the goal of mimicking complex biominerals. Eggshell biomimetics is a nascent field compared to other biominerals, therefore a reasonable strategy to unveil the mechanism of eggshell formation is to analyse the effect of organic additives one at a time, with and without an organic scaffold.

Because the proteins are too complex to be harnessed properly, a widely-accepted strategy is to use well-known polymers to mimic the role of proteins in biominerals. This method allows us to form a solution reminiscent of the biomineralization environment, which includes a liquid phase separation, and is hence called polymer-induced liquid precursor (PILP) process. The additive polymer acts as a precursor and triggers a phase separation in the solution and subsequently becomes the site of crystal growth. The PILP process has been shown to be promising as it is but without the regulation of proteins, the formation of bulk biomimetic minerals is still an elusive goal. Addressing this problem requires harnessing organic molecules, both as mineralization regulators and scaffolds.

Therefore, here we have used both the PILP process and a synthetic peptide, which is the binding domain of eggshell protein Ovocleidin, to analyse the effects of these organic molecules on $CaCO_3$ crystals in the presence and absence of a scaffold. The scaffold here is extracted eggshell collagen membrane. $CaCO_3$ was grown in water in glass petri dishes using equal volumes of $CaCl_2$ and $NaHCO_3$. Mineralization was conducted at room temperature for 24 hours. The final $CaCO_3$ concentration is 50 mM. Both for the PILP and Ovocleidin-inspired synthetic peptide, the final concentration of the additive is 100 μg ml^{-1}. In the case of PILP, poly-aspartic acid was used as the precursor polymer additive, and was added to Ca^{2+} solution before $CaCO_3$ mineralization. The synthetic peptide used in this study has an amino acid sequence of RPAGSRSWR, which is the binding domain of Ovocleidin to $CaCO_3$.

The peptide was added to the CO_3^{2-} solution as it is known that this domain interacts with CO_3^{2-} ions. Eggshell collagen membrane (Figure 1) was extracted by dissolving the shell of a chicken egg in 1 M HCl in 2 h at room temperature.

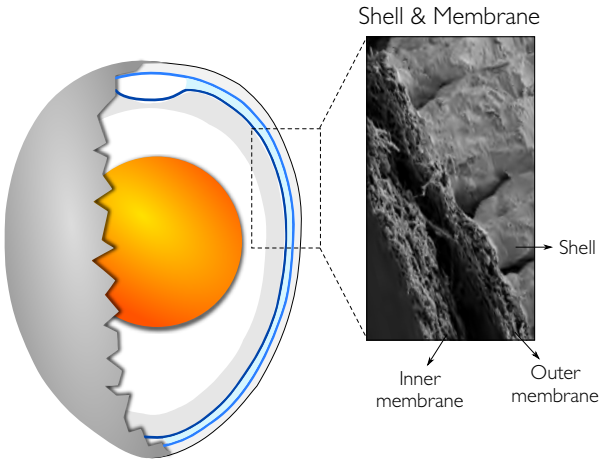

Figure 1: Cross section of an eggshell illustrating the direct contact of the shell with the outer shell membrane, a result of the membrane being the site of nucleation of biomineral.

The supernatant of the mineral solutions were discarded and the crystals were analysed with Scanning Electron Microscopy (SEM) immediately after sputter-coating of the samples with palladium. Figure 2 shows an SEM image of a single crystal of $CaCO_3$. As can be seen, the crystal has an equilibrium morphology as expected.

Figure 3 shows the crystals grown in the same conditions as in Figure 2 but this time using a collagen scaffold, the natural eggshell membrane. The obvious change in the morphology of the crystals can be attributed to the effect of the membrane. It is shown that even the crystals in close contact to each other present different morphologies. Non-equilibrium morphologies are also different from each other which suggests that the effect of the membrane is intriguingly different from point to point. This result shows two key factors regarding the eggshell membrane. Firstly, the presence of a membrane changes the morphology in different ways for each crystal. Secondly, it is challenging to control the morphology by only modifying the membrane, considering the presence of both equilibrium and non-equilibrium shapes adjacent to one another.

Figure 4 shows an SEM image of $CaCO_3$ crystals grown using a PILP additive. As in the case of any additive, the equilibrium morphology (Figure 2) no longer exists and spherical crystals are dominant with a homogenous distribution. A key difference is the presence of thin-film like structures as can be seen at the left side of the image. It is interesting that the change in morphology can be observed in a single SEM image. In the middle of the image, the formation of the thin film can be interpreted as follows: the formation of spherical crystals was followed by the merging of the crystals into a thin film. The absence of a scaffold shows the effect of the PILP process alone and it raises the question of whether the poly-aspartic acid triggers only the formation of spherical particles or if it also triggers them to merge.

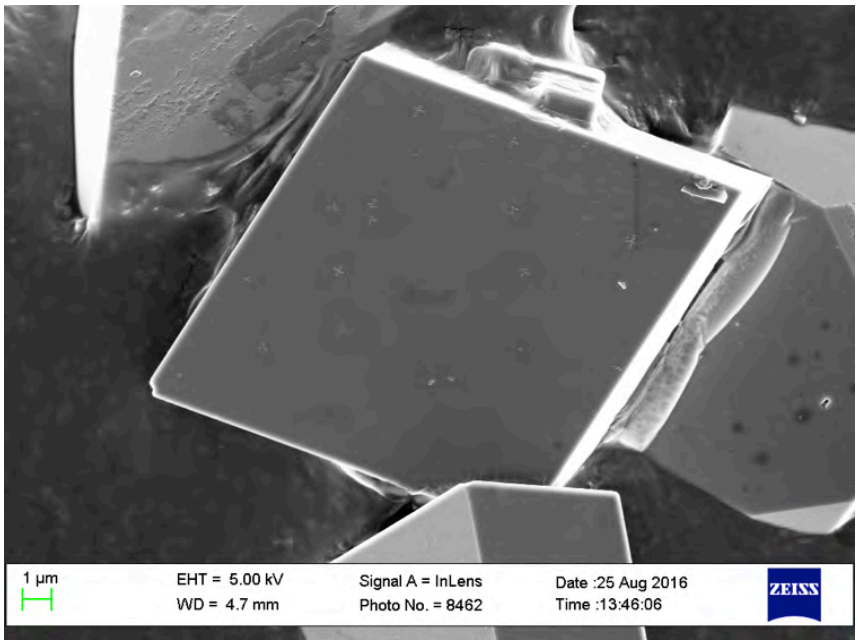

Figure 2: SEM image of $CaCO_3$ single crystal grown in water

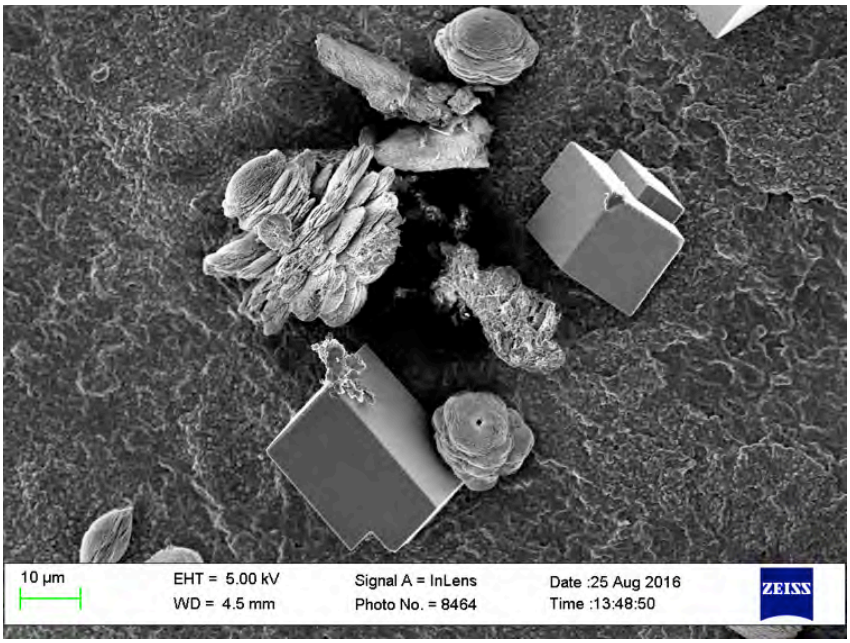

Figure 3: SEM image of $CaCO_3$ crystals grown in water on a collagen scaffold

Figure 5 shows an SEM image of $CaCO_3$ crystals grown using a PILP process on a collagen membrane. The difference between Figure 4 and Figure 5 reveals the importance of the eggshell membrane and of the PILP additive. Because there is both a scaffold and an additive, the crystals were expected to be modified by both. It is interesting that the $CaCO_3$ in this case presents a stark difference even from non-equilibrium morphologies that have been observed previously. The crystals seem

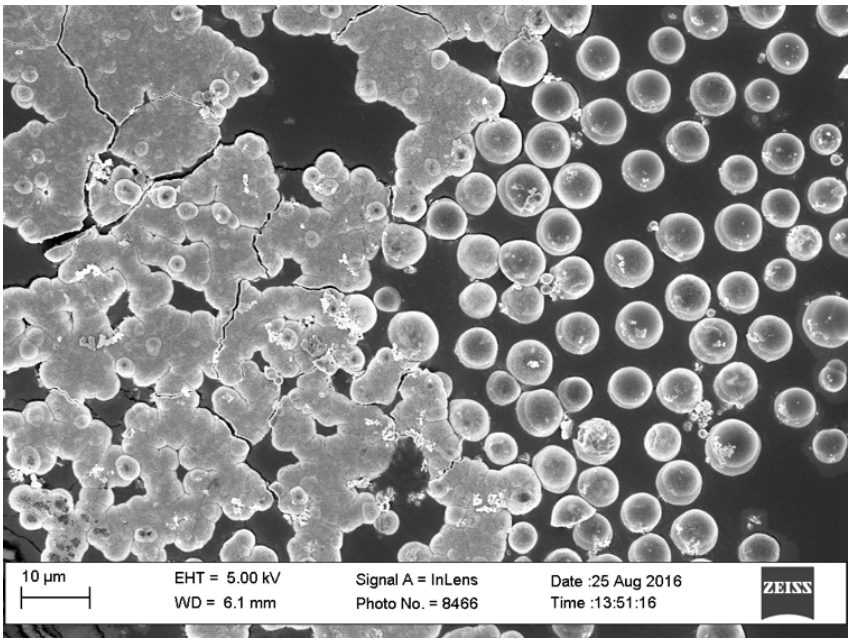

Figure 4: SEM image of $CaCO_3$ crystals grown in water using a polymer-induced liquid precursor (PILP) mineralization. Poly-aspartic acid was used as the PILP additive with a final concentration of 100 $\mu g\ ml^{-}1$

to have been merged in a disordered manner yet there is no similarity to the natural eggshell crystals, which resembles stretched rhombohedral crystals.

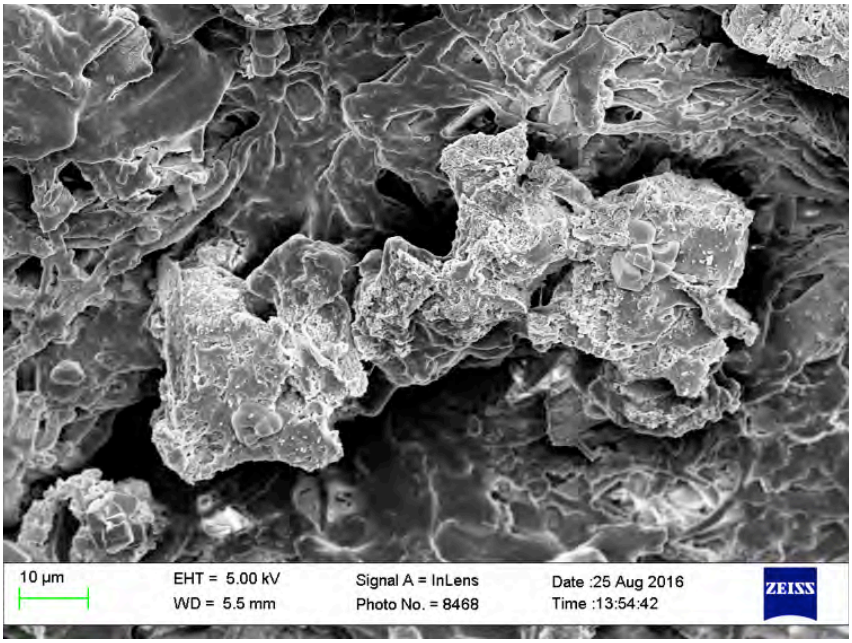

Figure 5: SEM image of $CaCO_3$ crystals grown in water using PILP on an eggshell collagen membrane

Figure 6 shows an SEM image of $CaCO_3$ crystals grown in the presence of a

synthetic peptide. Because there is no scaffold or any other additive in the mineralization solution, the merging effect can be attributed to the Ovocleidin-inspired synthetic peptide. The morphology of the crystals suggests that the peptide leads to merging of the crystals without much change in rhombohedral shape. This is reminiscent of the eggshell palisade layer where the crystals unilaterally grow in their c-axis without a non-equilibrium shape, unlike other natural biominerals, such as sea urchin spines.

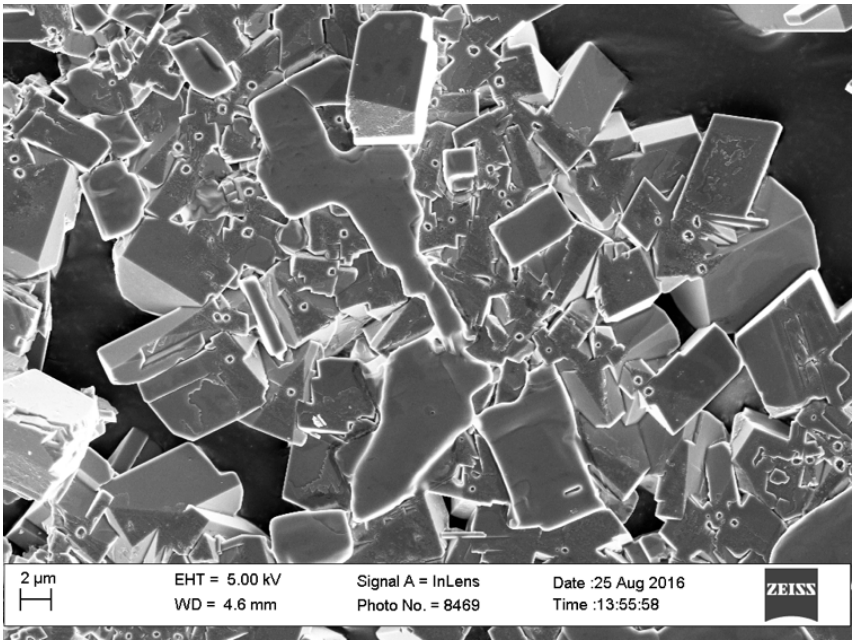

Figure 6: SEM image of $CaCO_3$ crystals grown in water using eggshell protein Ovocleidin-inspired synthetic peptide

Figure 7 shows the effect of the peptide on crystal growth in the presence of a collagen scaffold. In this case there are single crystals which have both equilibrium and non-equilibrium shapes and it is worth noting that the crystals do not tend to merge. This behavior suggests the importance of the sequence of events in crystal growth. In eggshell formation, crystal growth starts in organic spots on the collagen membrane. It is important to note that the mineralization regulator proteins are concentrated in these spots, which means that the first crystals are not in contact with the collagen membrane but rather they interact first with proteins, such as ovocleidin. Therefore, Figure 7 suggests that the growth of $CaCO_3$ crystals might start in the presence of an organic additive and then deposit on the scaffold as in the case of eggshell formation.

Figure 8 shows an SEM image of $CaCO_3$ crystals in the presence of both polyaspartic acid and Ovocleidin-inspired peptide. The existence of both thin films and crystals with non-equilibrium needle-like morphology shows that there is a combined effect that PILP process and peptide additive have on the growth of crystals. Although some crystals are reminiscent of the palisade layer in eggshell, the effect of PILP seems to be more dominant than that of the peptide here, as there is a tendency

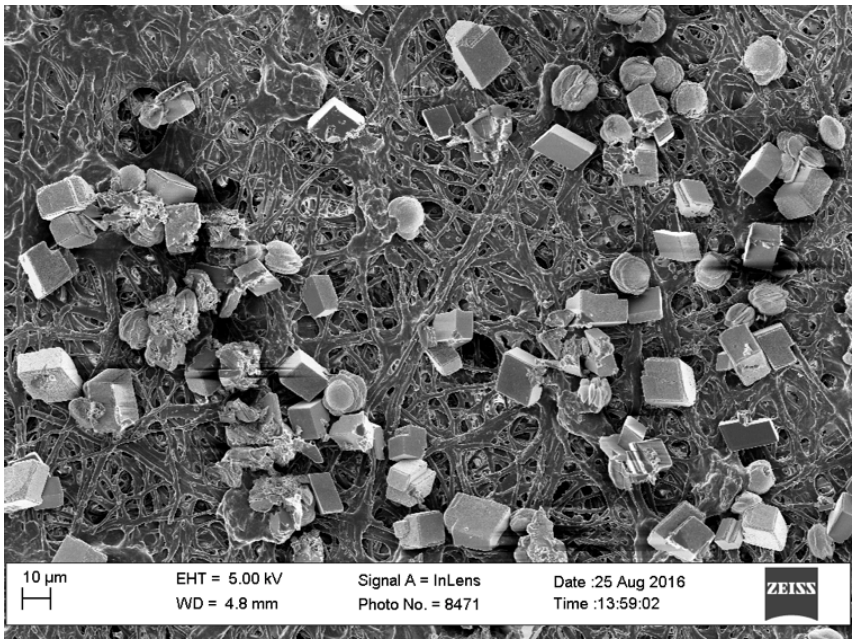

Figure 7: SEM image of $CaCO_3$ crystals grown in water using eggshell protein Ovocleidin-inspired synthetic peptide on eggshell collagen membrane

towards spherical crystals and thin film formation.

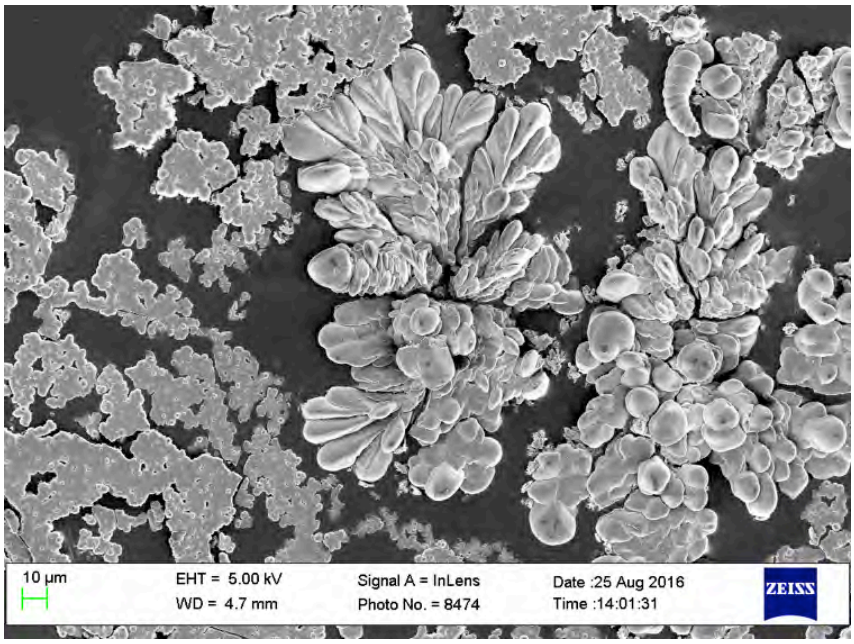

Figure 8: SEM image of $CaCO_3$ crystals grown in water in the presence Ovocleidin-inspired synthetic peptide and PILP additive poly-aspertic acid

Figure 9 shows an SEM image of $CaCO_3$ crystals grown in the presence of poly-aspartic and peptide on the collagenous eggshell membrane. In this case, there is a stark difference from all other conditions in the previous experiments (Figures 1-7).

Compared to Figure 8, the effect of collagen is of utmost importance. The combined effects of PILP and peptide on the collagen lead to the formation of large thin films throughout the specimen. (It is worth noting the scale of the image is 100 μm) There is neither an equilibrium morphology nor a non-equilibrium shape that was observed in other experiments. The circular thin films seem to merge while they grow and even build up on another as can be seen in the bottom left of the image. Although this formation looks different from the natural eggshell palisade layer, where crystals grow unilaterally upwards, it can be said that this type of formation is a step closer to the bulk formation of biominerals even if without having a complex shape.

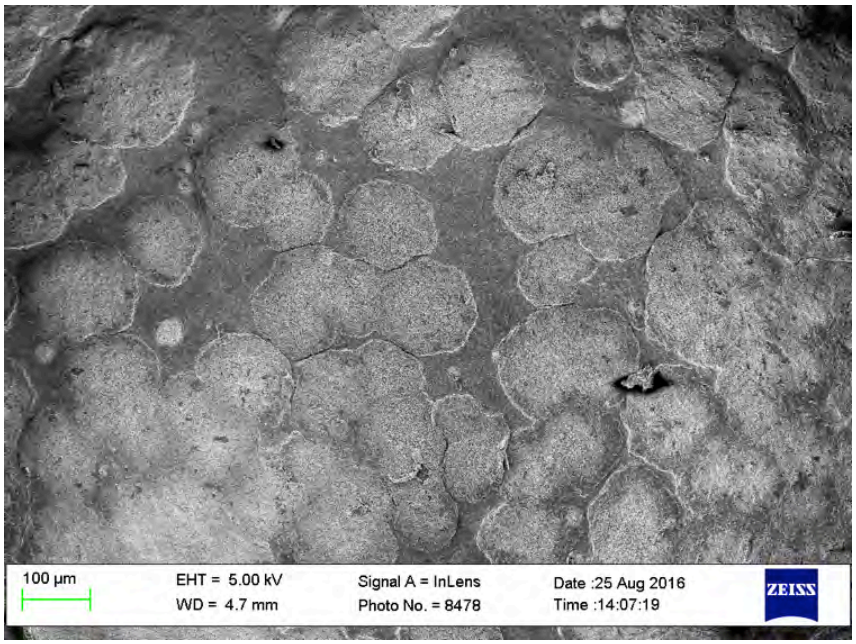

Figure 9: SEM image of $CaCO_3$ grown in water in the presence Ovocleidin-inspired synthetic peptide and PILP additive poly-aspertic acid on eggshell collagen membrane

This section has been a systematic study of eggshell biomineralization with three different factors in different combinations: (1) glass versus collagenous eggshell membrane as the mineralization substrate; (2) the presence or absence of the PILP additive poly-aspartic acid; (3) the presence or absence of a synthetic peptide mimicking the natural eggshell protein Ovocleiden. These three different factors clearly interact in complex ways, and these intriguing interactions are worthy of study in significantly more detail in future studies.

The Shell Membrane in Eggshell Fracture

Because of the importance of the eggshell membrane (Figure 1) as the site of nucleation in eggshell biomineralization, it was hypothesized that this membrane might play a mechanical role. Therefore, an experiment was designed and executed with the aim of trying to elucidate whether the shell membrane has a mechanical role. Eggs

were obtained from the local supermarket and holes were punctured at the equator in order to remove the liquid contents (both yolk and white). Eggs were tested in this condition, denoted as "untreated", in a commercial small-force Instron universal test frame (Canton, MA, USA) with the egg resting on a transparent platform to allow for filming from underneath the sample in order to visualize cracking (Figure 10).

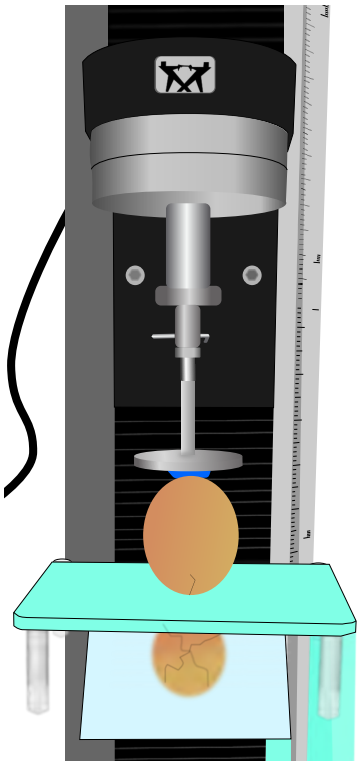

Figure 10: Experimental set-up for whole egg fracture tests. The egg rests on a custom transparent platform to allow for filming from underneath.

For each egg tested, two quantitative analyses were considered. The peak force was recorded, and was always an unambiguous point (Figure 11). Also the area under the force-displacement curve was numerically integrated to calculate the mechanical work done to failure. Mechanical load-displacement graphs were normalized, force to the peak force and displacement to the total egg height. The normalized load-displacement responses generally appeared as in Figure 11, with a sharp rise to maximum force, followed by a steep drop off in force and a jagged response centred around 0.2-0.4 P_{max}. The egg appearance for untreated control eggs is shown in FIgure 12, with a "bullseye" type fragmentation pattern with many fragments on the order of a few mm. The failure pattern actually resembles "mud-flat cracking" where a brittle film situated on a ductile substrate fragments into relatively uniform fragments. Here the shell membrane is acting as the ductile substrate, and importantly, the fragments remain attached to the membrane. This is intuitively sensible since the shell nucleates on the membrane, so the fact that they are and remain mechanically connected is logical.

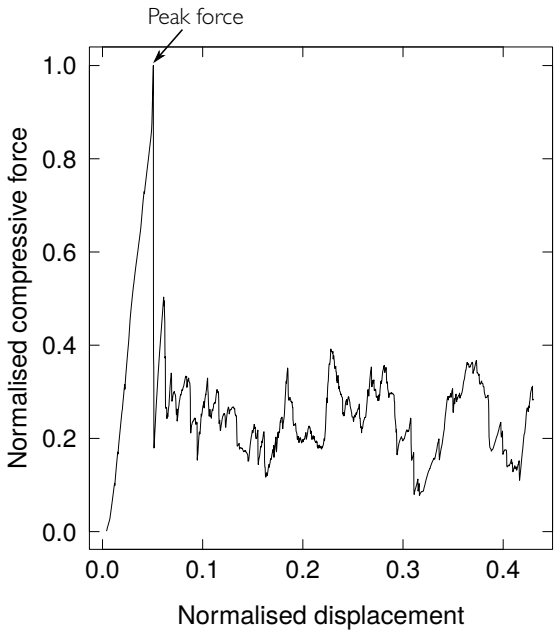

Figure 11: Normalized compressive force-displacement data for whole egg fracture.

Figure 12: Photograph of typical fracture pattern of an intact egg.

In order to manipulate the membrane, and see what effect this has on the mechanical performance of the egg, chemical treatments of the membrane were performed to obtain eggs with two altered membrane conditions: after filling and soaking the egg interior with sodium hypochlorite (NaOCl, common bleach) to dissolve the proteinous collagen shell membrane, denoted as "membrane removed", or after filling and soaking the egg interior with acetone to dehydrate the proteinous collagen shell membrane, denoted as "membrane dried". Tests were performed on $n = 16$ eggs for each of the three conditions, untreated, membrane removed, and membrane dried.

Figure 13 shows the peak force at fracture initiation for the three membrane conditions, illustrating that alteration of the membrane gives rise to no substantial difference. This was not the case for the work of fracture (Figure 14), which demonstrates that the membrane has a substantial effect. Removal of the membrane results in about a 70% decrease in the total fracture work, and the morphology of the cracking was different as well: the fragments were large and the fracture was much more brittle in that the normalized displacement was much less. In other words, the membrane is contributing to the breaking resistance of the overall egg by assisting with energy absorption as opposed to more immediate destruction of the membrane-less egg. In the third condition, where the membrane was present but severely dehydrated, an intermediate result was obtained for the work of fracture.

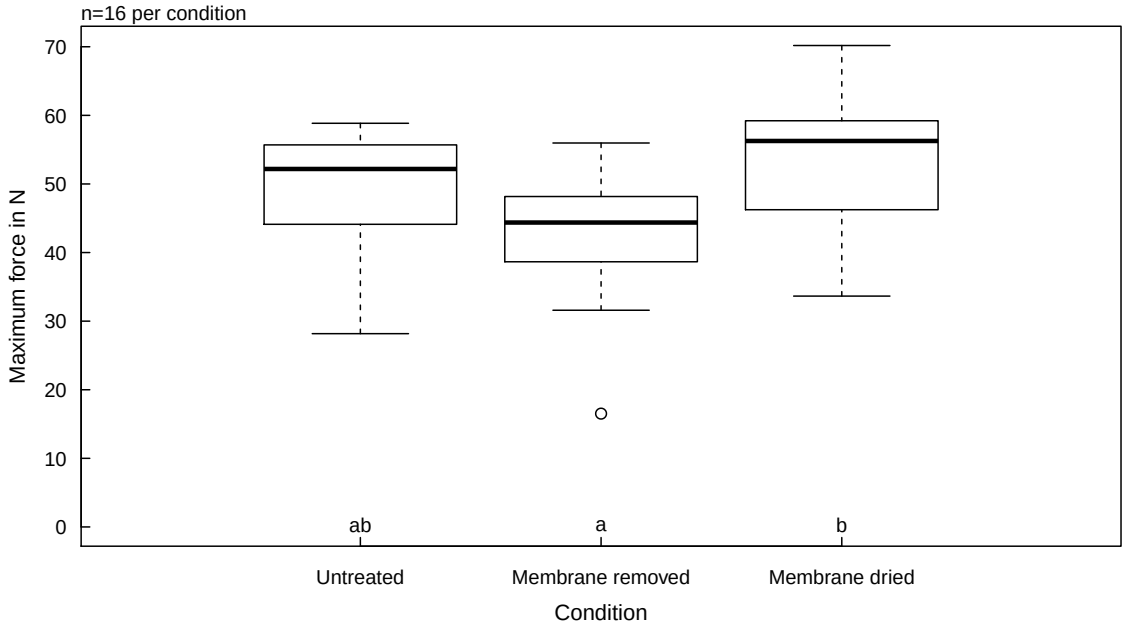

Figure 13: Peak force at fracture initiation for intact (untreated) egg, egg with the shell membrane removed, and egg with the shell membrane in place but dehydrated.

Thus, in this study we have demonstrated the importance of the eggshell membrane in the mechanical deformation resistance of the entire egg. The fragments in untreated eggs remain firmly attached to the membrane even as the shell itself fractures. Removal of the membrane or dehydration of the membrane in place both make the egg less resistant to fracture, but the membrane-free case is the more extreme situation, resulting in near brittle fracture of the egg with much larger shell fragments. One question that remains is what the quantitative fracture resistance of the shell material itself actually is. These tests were structural, not aimed at material properties measurement. Investigation of the literature illustrates that this remains an open question, such that fracture resistance measurements of eggshell calcite, with its protein inclusion, remains an open area for future research.

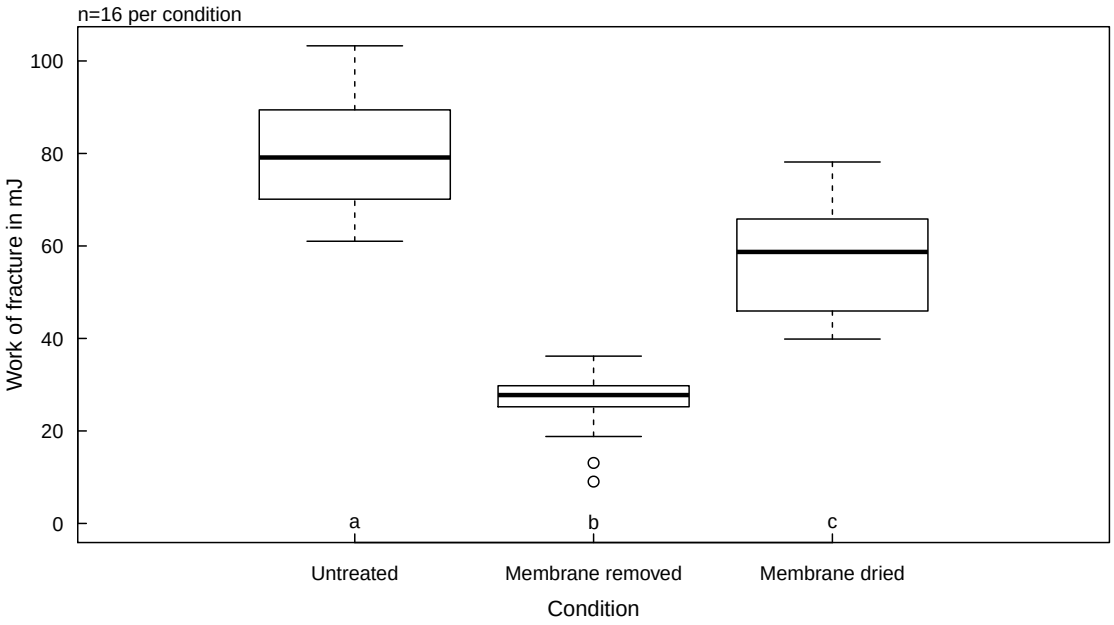

Figure 14: Work of fracture for intact (untreated) egg, egg with the shell membrane removed, and egg with the shell membrane in place but dehydrated.

Conclusion and Outlook

The two-year pilot project has made significant advances in understanding eggshell biomineralization, with an aim to scaling up the bulk production of biomimetic eggshell for structural applications. It is anticipated that although not yet ready for submission, at least four research manuscripts will be prepared from the work contained within the three reports:

- Organic molecule inclusion in calcite. This includes the TGA thermal analysis from the preliminary report, FTIR data from both the preliminary and intermediate reports, and the organic molecule entrapment assay from the intermediate report.
- Comparative eggshell mechanics across species. This will be an expansion of the nanoindentation and SEM studies on multiple species' shells from the intermediate report, expanded to include even more species via a collaboration with a zoo, who are providing eggshell pieces for analysis.
- Whole egg and membrane mechanics. This will be a full expansion of the work in the second half of this report.
- Biomineralization effects of eggshell membrane, poly-aspartic acid via PILP, and synthetic eggshell-like peptide on calcite formation. This will be a full expansion of the work in the second half of this report.

A further element of the work done to date is the production of an artificial eggshell membrane based on electrospun gelatin scaffolds contact printed with eggshell proteins. This work is on hold, as there was an unfortunate electrical fire in our laboratory this summer, which took out both of our electrospinning rigs. We anticipate getting the insurance settlement and getting back to work on this aspect in the coming months.

The eggshell work will continue as part of task 1 of the cooperative research agreement between the University of Cambridge and ERDC, "Synthesis and Characterization of Natural and Bio-inspired Materials" commencing immediately. The other two tasks in that project concern electrospun fiber composites and nanoindentation of hydrated materials. This three year project has just commenced and will be the subject of future reports.